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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

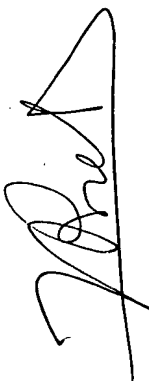
In re application of: Jesus Prieto VALTUENA, et al
Serial No.: 09/674,445 Group No.: 1614
Filed: November 1, 2000 Examiner.: Jegatheesan Seharaseyon
For: UTILIZATION OF INTERFERON ALPHA 5 IN THE TREATMENT OF
VIRAL HEPATOPATHIES

Attorney Docket No.: U 013039-2

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 CFR 1.132

I, Jesús Prieto, hereby declare:

- 
1. I am one of the co-inventors of the subject matter described and claimed in the above application. I make this declaration in support of the application. A copy of my curriculum vitae is annexed hereto as Exhibit 1.
 2. I have recently co-authored a paper (sent for publication) that describes experimentation that compares the specific antiviral action in liver of IFN alpha 5 with another well known interferon subtype (IFN alpha 2) broadly used in antiviral therapy of HCV. A copy of the paper is annexed hereto as Exhibit 1. I conducted or supervised the experimentation described in the paper and can attest that the reported results are accurate.
 3. The results of the experimentation described in the paper show that the antiviral action attributed to IFN alpha 5, measured as cell signaling and antiviral gene induction, was more efficient and intense than when IFN alpha 2 was used. Although only HCV therapy is exemplified in the paper, the role of liver natural defense of IFN alpha 5 would not be expected to differ in other liver diseases of viral origin.
 4. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and

further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity or the application of any patent issued thereon.

Date: 23, 09.03

JESUS PRIETO VALTUEÑA

Name:





BIOGRAPHICAL SKETCH

NAME PRIETO, JESUS	POSITION TITLE
BIRTH: Oviedo (Spain) April,6 1944 [DNI:10486228]	PROFESSOR

EDUCATION AND TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR	FIELD OF STUDY
University of Valladolid (Spain)	M.D.	1967	Medicine
University of Valladolid	Ph. D.	1969	Medicine (Hepatology)
University Hospital of Valladolid	Board Certifications in Gastroenterology and Internal Medicine	1969 1970	Gastroenterology/Int. Medicine
Royal Free Hospital. London (Prof. Sheila Sherlock)	Post Doctoral Studies and Clinical Assistant	1972-73	Hepatology

PROFESSIONAL EXPERIENCE

INSTITUTION AND LOCATION	TITLE	YEAR	FIELD
University Hospital of Valladolid (Spain)	Assitant Professor and Clinical Assistant	1970-72 1974-75	Int.Medicine/ Gastro/Hepatol
University Hospital of Valladolid	Associate Professor	1976	Medicine
University of Oviedo (Spain)	Professor of Medicine	1976-77	Medicine
University of Santiago de Compostela and General Hospital of Galicia (Spain)	Professor of Medicine and Chairman Department of Medicine	1977-79	Int. Medicine/Gastro/Hepatol.
University of Navarra and Clinica Universitaria de Navarra (Spain)	Professor of Medicine co- Chairman Department of Medicine and Liver Unit	1979-85	Int. Medicine/Gastro/Hepatol
University of Navarra and Clinica Universitaria de Navarra	Professor of Medicine Chairman Department of Medicine and Liver Unit	1985-1996	Int. Medicine/Gastro/Hepatol
University of Navarra and Clinica Universitaria de Navarra	Professor of Medicine Chairman Department of Medicine and Chief Division of Hepatology and Gene Therapy	1997-	Int. Medicine/Gastro/Hepatol

AWARDS AND HONORS

- President of the Spanish Association for the Study of the Liver (2001-)
- Vice-President of the Spanish Association for the Study of the Liver (1985-89)
- President of the Society of Internal Medicine of Navarra, Aragon and Basque Country (1993-94)
- Member of the Scientific Committee of the European Association for the Study of the Liver (1989-92)
- Doctor Honoris Causa . Faculty of Medicine. University of Porto (Portugal)
- Founder of the Spanish Society of Gene Therapy (2000)
- Member of the Scientific Board of ANRS (Agence Nationale Française pour la Recherche sur le SIDA et l'Hépatite C- National French Agency for Investigation on AIDS and Hepatitis C) 2000-
- Expert of INSERM (Institut National Français pour la Santé et la Recherche Médicale; National French Institute for Health and Medical Research) 2000-
- Member of the Committee of Experts of the Spanish Ministry of Health for evaluation of Interferon therapy. 1998
- Expert of the Spanish Ministry of Health for evaluation of new drugs (Agencia Española del Medicamento) 2000-
- Member or ex- member of the Editorial Committee of Hepatology Research, Journal of Hepatology, Alimentary Tract Pharmacology and Therapeutics, Revista Clínica Española, Medicina Clínica, Hepatología y Gastroenterología, Revista Española Enfermedades del Aparato Digestivo.
- Award "Candida Medrano de Merlo" for the work on "Gene Therapy of Liver Cancer"
- Awarded Spanish patent for the use of Interferon alpha 5 for the treatment of liver diseases of viral and neoplastic origin. P 980 1003 (BOE 16/Aug/2000) (pending the European patent)
- Several papers were commented in different issues of *Year Book of Medicine* and deserved editorials in New England Journal of Medicine, Gastroenterology, Hepatology, Gut and other journals.
- Invited speaker in international symposia and meetings of different national Societies of Hepatology and Gastroenterology such as: Meeting of the French Association for the Study of the Liver (Paris, 1993), European Association for the Study of the Liver (Naples, 1999), Spanish Society of Gastroenterology (Madrid 1997), Asian Symposium on Liver Diseases (Beijing 1999), European Gastroenterological Week (Brussels, 2000), Italian Association for the Study of the Liver (Rome,

2001), American Association for the Study of the Liver (Single Topic Conference, Airlie, Virginia, 2001), British Association for the Study of the Liver (London 2001), Polish Association for the Study of the Liver (Mikolajki, Poland, 2001).

PUBLICATIONS

1. "Die Elimination von Bromosulphalein (BSP): Mathematische Untersuchung des Verhaltens dieser Substanz bei intravenöser Anwendung in Einer Einzeldosis". J. Prieto Valtueña, T. Calvo del Olmo, S. de Castro del Pozo. **ACTA HEPATO-GASTROENTEROLOGICA** 1972; 19: 352.
2. "Serum Ferritin in Patients with Iron Overload and with Acute and Chronic Liver Disease". J. Prieto, M. Barry and S. Sherlock. **GASTROENTEROLOGY** 1975; 68: 525.
3. "Serum Ferritin Assay and Iron Status in Chronic Renal Failure and Haemodialysis". S. Hussein, J. Prieto, M. O'Shea, A.V. Hoffbrand, R.A. Baillod, J.F. Moorhead. **BRITISH MEDICAL JOURNAL** 1975; 1: 546.
4. "Immune complexes in epidemic (type A) hepatitis. Detection by three methods using laser nephelometry". M. Serrano, J. Prieto, J.M. Esteban and C.D. Crisci. **ALLERGOLOGY ET IMMUNOPATHOLOGY** 1981; 9,5: 433-440.
5. "Mediation of a receptor mechanism in the uptake of iron from transferrin by the hepatocyte". R.M. Nunes, J.M. Prieto and B.J. Potter. **PROTIDES OF THE BIOLOGICAL FLUIDS 29th COLLOQUIUM** 1981. Edited by H. Peeters. Pergamon Press. Oxford and New York, pag. 455-458, 1982.
6. "Serum Antibodies Against Porphyrin Hepatocytes in Patients with porphyria cutanea tarda and liver disease". E. Baravalle and J. Prieto. **GASTROENTEROLOGY** 1983; 84: 1483-1491.
7. "Intracerebroventricular infusion of sodium chloride-rich artificial cerebrospinal fluid in rats induces natriuresis and releases an inhibitor of prostaglandin synthesis". J. Díez, I. Colina, F. Guarner, J. Quiroga, J. Corzo, A. Purroy and J. Prieto. **CLINICAL SCIENCE** 1984; 66: 621-624.
8. "Cytoprotective effect of prostaglandins on isolated rat liver cells". F. Guarner, M. Fremont-Smith and J. Prieto. **LIVER** 1985; 5: 35-39.

9. "Increased Synthesis of Systemic Prostacyclin in Cirrhotic Patients". F. Guarner, C. Guarner, J. Prieto, I. Colina, J. Quiroga, J. Casas, R. Freixa, J. Roselló, E. Gelpi and J. Balanzó. **GASTROENTEROLOGY** 1986; 90: 687-694.
10. "Renal prostaglandins in cirrhosis of the liver". C Guarner, I. Colina, F. Guarner, J. Corzo, J. Prieto and F. Vilardell. **CLINICAL SCIENCE** 1986; 70: 477-484.
11. "Effect of Spironolactone on Renal Prostaglandin Excretion in Patients with Liver Cirrhosis and Ascites". J.F. Medina, J. Prieto, F. Guarner, J. Quiroga and A. Milazzo. **JOURNAL OF HEPATOLOGY** 1986; 3: 206-211.
12. "Interleukins in chronic active hepatitis B: Relationship with viral markers". Maria Pilar Civeira, Jesús Prieto, Susana Morte, Marta Riñón and Manuel Serrano. **JOURNAL OF HEPATOLOGY** 1987; 5: 37-44.
13. "Monocyte Function in Chronic Non-A, Non B Hepatitis: Relationship with the Activity of Liver Disease". A. Castilla, M. Serrano, S. Morte, M.L. Subirá, M.P. Civeira and J. Prieto. **VIRAL HEPATITIS AND LIVER DISEASE** 1988; Alan R, Liss, Inc., pág. 568-571.
14. "Gamma-Interferon Production by Peripheral Mononuclear Cells in Patients With Chronic Non-A, Non-B Hepatitis". M. Serrano, S. Morte, A. Castilla, M.P. Civeira and J. Prieto. **VIRAL HEPATITIS AND LIVER DISEASE** 1988; Alan R, Liss, Inc., pag. 572-575.
15. "Opioid peptides modulate the organization of vimentin filaments, the phagocytic activity and the expression of surface molecules in monocytes". J. Prieto, M.L. Subirá, A. Castilla, J.L. Arroyo, M. Serrano. **SCANDINAVIAN JOURNAL OF IMMUNOLOGY** 1989; 29: 391-398.
16. "Cytoskeletal Organization and Functional Changes in Monocytes from Patients with Chronic Hepatitis B: Relationship with Viral Replication". J. Prieto, A. Castilla, M.L. Subirá, M. Serrano, S. Morte and M.P. Civeira. **HEPATOLOGY** 1989; 9 (5): 720-725.
17. "Naloxone-Reversible Monocyte Dysfunction in Patients with Chronic Fatigue Syndrome". J. Prieto, M.L. Subirá, A. Castilla and M. Serrano. **SCANDINAVIAN JOURNAL OF IMMUNOLOGY** 1989; 30: 13-20.

18. "Systemic and Regional Hemodynamics in Patients With Liver Cirrhosis and Ascites With and Without Functional Renal Failure". J. Fernández-Seara, J. Prieto, J. Quiroga, JM. Zozaya, MA. Cobos, JL. Rodríguez-Eire, A. García-Plaza and J. Leal. **GASTROENTEROLOGY** 1989; 97: 1304-1312.
19. "Monocyte disorder causing cellular immunodeficiency: a family study". J. Prieto, M.L. Subirá, A. Castilla, M.P. Civeira and M. Serrano. **CLINICAL EXPERIMENTAL IMMUNOLOGY** 1990; 79: 1-6.
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24. "Abnormal Sympathetic and Renal Response to Sodium Restriction in Compensated Cirrhosis". M.A. Simón, J. Diez and J. Prieto. **GASTROENTEROLOGY** 1991; 101: 1354-1360.
25. "Detection of hepatitis C virus antibodies with new recombinant antigens: assessment in chronic liver diseases". J.I. Riezu-Boj, D. Parker, M.P. Civeira, D. Phippard, T.P. Corbishley, J. Camps, A. Castilla and J. Prieto. **JOURNAL OF HEPATOLOGY** 1992; 15: 309-313.
26. "Hepatitis B and C viral infections in patients with hepatocellular carcinoma". J. Ruiz, B. Sangro, JI. Cuende, O. Beloqui, JI. Riezu-Boj, JI. Herrero and J. Prieto. **HEPATOLOGY** 1992; Vol. 16, No. 3: 637-641.

27. "Replication of hepatitis C virus in peripheral blood mononuclear cells: effect of alpha-interferon therapy". C. Qian, J. Camps, MD. Maluenda, MP. Civeira and J. Prieto. **JOURNAL OF HEPATOLOGY** 1992; 16: 380-383.
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31. "Partial Splenic Embolization for the Treatment of Hypersplenism in Liver Cirrhosis". B. Sangro, I. Bilbao, I. Herrero, C. Corella, J. Longo, O. Beloqui, J. Ruiz, JM. Zozaya, J. Quiroga, J. Prieto. **HEPATOLOGY** 1993; 18 (2): 309-314.
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34. "Randomised trial of lymphoblastoid alfa-interferon in chronic hepatitis C. Effects on inflammation, fibrogenesis and viremia". J. Camps, A. Castilla, J. Ruiz, MP. Civeira and J. Prieto. **JOURNAL OF HEPATOLOGY** 1993; 17: 390-396.
35. "Hepatitis B virus occult infection in subjects with persistent isolated anti-HBc reactivity". A. Sánchez-Quijano, J.I. Jauregui, M. Leal, J.A. Pineda, A. Castilla, M.A. Abad, M.P. Civeira, F. García de Pesquera, J. Prieto and E. Lissen. **JOURNAL OF HEPATOLOGY** 1993; 17: 288-293.

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41. "Prediction of sustained remission of chronic hepatitis C after a 12-month course of alfa interferon". J. Camps, M. García-Granero, J.I. Riezu-Boj, E. Larrea, E. de Alava, M.P. Civeira, A. Castilla, J. Prieto. **JOURNAL OF HEPATOLOGY** 1994; 21: 4-11.
42. "Atrial natriuretic factor in cirrhosis: relationship to renal function and hemodynamic changes". C. Fernández-Rodríguez, J. Prieto, J. Quiroga, J.M. Zozaya, A. Andrade, D. Rodríguez-Martínez. **JOURNAL OF HEPATOLOGY** 1994; 21: 211-216.
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44. "Plasma Levels of Substance P in Liver Cirrhosis: Relationship to the Activation of Vasopressor Systems and Urinary Sodium Excretion". C.M. Fernández-Rodríguez, J. Prieto, J. Quiroga, J.M. Zozaya, A. Andrade, M. Núñez, B. Sangro, J. Penas. **HEPATOLOGY** 1995; 21 (1): 35-40.

45. "Anion Exchanger Immunoreactivity in Human Salivary Glands in Health and Sjögren's Syndrome. J.J. Vázquez, M. Vázquez, M.A. Idoate, L. Montuenga, E. Martinez-Ansó, J.E. Castillo, N. García, J.F. Medina, J. Prieto. **AMERICAN JOURNAL OF PATHOLOGY** 1995; 146 (6): 1422-1432.
46. "Induction of Sensitivity to Ganciclovir in Human Hepatocellular Carcinoma Cells by Adenovirus-Mediated Gene Transfer of Herpes Simplex Virus Thymidine Kinase". Ch. Qian, R. Bilbao, O. Bruña, J. Prieto. **HEPATOLOGY** 1995; 22 (1): 118-123.
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- Chemello, M.P. Civeira, C. Frost, G. Dusheiko. **JOURNAL OF HEPATOLOGY** 1996; 24: 517-524.
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- 1994:-1996 “Development of new strategies to treat Chronic Hepatitis B:Use of *marmota monax* as a animal model” DGCICY, PB93-1227
- 1995-1997: “Gene Therapy of Hepatocellular Carcinoma using Suicide Genes” (Fundacion Echebano)
- 1999-2002: “Prevention and therapy of woodchuck hepatitis virus infection using immunization with defective recombinant adenoviruses and gene transfer by means of gene gun” CICYT. SAF 99-0084
- 1999-2001: Biological effects of Interferon alpha 5 : interaction with hepatitis C virus (Fundacion Echebano)
- 2000-2003: “Targeted vectors for cancer gene therapy: receptor and transcriptional targeting of retroviral, lentiviral, and adenoviral vectors”. European Commission. QLK3-CT-1999-00364
- 2000-2002: “Gene Therapy of neoplastic and viral diseases of the liver” Fundación Ramon Areces



Interferon- α 5, the interferon- α subtype produced in the liver, signals stronger and induces higher expression of interferon-inducible genes in hepatocytic cells than interferon- α 2

Esther Larrea*, Rafael Aldabe*, Jose-Ignacio Riezu-Boj, Anunciata Guitart, Maria Pilar Civeira, Jesus Prieto and Elena Baixeras,

Division of Hepatology and Gene Therapy, Department of Medicine, Clínica Universitaria, University of Navarra, Pamplona, Spain

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- Esther Larrea and Rafael Aldabe contributed equally to this work.

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Address reprint request to: Jesús Prieto, M.D., Division of Hepatology and Gene Therapy, Department of Medicine, Clínica Universitaria, University of Navarra, 31008 Pamplona, Spain. E-mail: jprieto@unav.es; fax (34) 948 296785.

Abbreviations: IFN, interferon; IFNAR, interferon alpha receptor; STAT, signal transducer and activator of transcription; PKC- δ , protein kinase C delta; ISRE, IFN-stimulated response element; IRF, IFN-regulatory factor; 2'-5' OAS, 2'-5' oligoadenylate synthetase; mRNA, messenger RNA; HCV, hepatitis C virus; PCR, polymerase chain reaction; Jak, janus kinase; Tyk, tyrosine kinase; GADPH, glyceraldehyde-3phosphate-dehydrogenase.

ABSTRACT

Interferon (IFN)- α 5 is the only IFN- α subtype expressed constitutively in the human liver suggesting a specific role of this subtype in liver cells. However, IFN- α 2, and not IFN- α 5, constitutes the basis of the currently used antiviral therapy of chronic viral hepatitis. IFN- α 2-based therapies fail to control viral replication in a great proportion of patients making necessary the introduction of more effective treatments for viral hepatitis. In this work we have compared IFN- α 2 and IFN- α 5 with respect activation of cell signaling cascades and induction of antiviral genes in hepatocytic cells. We found that the Tyr⁷⁰¹ phosphorylation of STAT1 at 15, 30 and 60 minutes after stimulation with IFN was two times stronger when cells were incubated with IFN- α 5 than when using IFN- α 2. Similarly tyrosine phosphorylation of the receptor-associated kinase Tyk-2 at 10 and 30 minutes after exposure to IFN was 4-8 times more intense when using IFN- α 5 than when using IFN- α 2. Moreover, Tyr⁷⁰⁵ phosphorylation of STAT3 was 1.5-2 times higher with IFN- α 5 than with IFN- α 2 at 1, 5, 15, 30 and 60 min after stimulation with IFN. In parallel to these findings, the mRNA levels of the antiviral IFN-inducible genes 2'-5' oligoadenylate synthase, p56 and MxA at 14h after incubation with IFN were about two times higher with IFN- α 5 than with IFN- α 2. In conclusion, our data show that IFN- α 5 produces stronger activation of cell signaling and more efficient induction of IFN-

HCV genotype 1 infection,¹⁴ the most prevalent genotype in the population. On the other hand IFN- α 2-based therapy of chronic hepatitis B induces sustained virological response in less than 40% of HBe positive patients and in less than 30% of HBe negative cases.¹⁵ Therefore, new therapies are urgently needed for all forms of chronic viral hepatitis.

IFN- α 5, being the IFN- α subtype expressed in the hepatic tissue, might represent an alternative therapeutic agent for viral infections affecting the liver. In the present paper we show that IFN- α 5 induces stronger activation signals and higher expression of antiviral genes than IFN- α 2 in hepatocytic cells, suggesting differences in the interaction with the liver cell receptor. Our data offer grounds for consideration of IFN- α 5 as a possible alternative therapeutic agent for chronic viral hepatitis.

MATERIAL AND METHODS

Cell culture. HepG2 human hepatoma cells (ATCC HB-8065) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, Maryland) supplemented with penicillin (0.6 μ g/ml), streptomycin (60 μ g/ml) glutamine (2mM) and 10% fetal calf serum.(FCS).

Stimulation of cells with IFN. IFN- α 2b was obtained from Schering-Plough (Madrid, Spain). Recombinant IFN- α 5 was kindly provided by Dr. Vytautas Naktinis (Biotechna, Vilnius, Lithuania). HepG2 cells were seeded at 200.000 /well in 6 well plates in DMEM 10% FCS. For signal transduction analyses, cells were serum-starved for 8 h prior to IFN exposure. IFNs were used at 5000 U/ml in the presence of 2% FCS for periods indicated in each experiment.

Antibodies. Anti-phospho-STAT1^{tyr701}, anti-phospho-STAT3^{tyr705}, anti-phospho-Tyk-2^{tyr1054/1055} antibodies and anti-Rabbit IgG HRP-linked antibody were purchased from Cell Signaling Bio-lab (Beverly, MA.). Anti-STAT3 antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-STAT1 antibody was from Santa Cruz Biotech, Inc. Anti-actin and anti-Tyk-2 antibodies were from Sigma-Aldrich (Steinheim, Germany) and Transduction Labs (Lexington, KY) respectively.

Western blotting . After trypsinization, cells were collected by centrifugation. Cell pellets were resuspended and lysed in sample buffer containing dithiothreitol. Samples (50 μ g protein) were resolved in SDS-PAGE under reducing conditions. Proteins were transferred onto nitrocellulose membranes (Bio-Rad laboratories, Inc) and stained with Ponceau red solution (Sigma-Aldrich,

sensitive genes than IFN- α 2 in hepatocytic cells. These results support the consideration of IFN- α 5 as a candidate therapeutic agent for chronic viral hepatitis.

INTRODUCTION

Interferons (IFNs) are a group of cytokines with pleiotropic effects including inhibition of cellular proliferation, induction of differentiation, modulation of the immune response and activation of an antiviral status in the cell.^{1,2} Human type I IFNs include a multigene family of different IFN- α subtypes and a single IFN- β . All type I IFNs are structurally related and share the same IFN receptor which is constituted at least by two subunits: IFNAR1 and the full-length IFNAR2c form.¹ The diverse activities of type I IFNs are mediated by conserved signal transduction pathways.^{3,4} Binding of IFN α/β to its receptor, triggers signals that are transmitted through signal transducers and activators of transcription (STATs) from cell surface receptor to the nucleus. Stimulation with IFNs- $\alpha/-\beta$ leads to tyrosine phosphorylation of the Jak-1 and Tyk-2 receptor-associated kinases. These two Janus kinases are responsible for the rapid activation of STATs. Indeed, Jak-1 phosphorylates Tyr⁷⁰¹ in STAT1 and Tyr⁶⁹⁰ in STAT-2 which form an oligomeric complex called ISGF3 also containing a third protein p48, a DNA binding protein. Tyrosine phosphorylation of STAT1 and STAT2 in response to IFN α/β occurs in all non-transformed cells. Full activity of STAT1 (isoform STAT1 α) requires phosphorylation on Ser⁷²⁷ probably via PKC- δ /p38 mitogen-activated protein kinase pathway.^{5,6,7} Then, ISGF3 translocates to the nucleus and activates the transcription of genes containing IFN-stimulated response elements (ISREs). IFN α/β also promote the formation of STAT1 homodimers, which bind to IFN γ activation sequence (GAS).⁸ Activation of Tyk-2 kinase, is essential to phosphorylate Tyr⁷⁰⁵ in STAT3 and soon this factor is phosphorylated in Ser⁷²⁷ to be fully activated.^{9,10,11} Tyrosine phosphorylation of STAT3, STAT4, STAT5 and STAT6 by type I IFNs takes place in a cell-type-specific manner.⁴ Translocation of these transcription factors to the nucleus culminates in the activation of IFN type I-sensitive genes.⁴ Of these genes, some are associated with regulation of apoptosis (caspases, Fas, p53), others with cell cycle arrest (p21, IFN-regulatory factor [IRF]-1), others with antiviral response (MxA, 2'-5' Oligoadenylate Synthetase (2'-5' OAS) and p56 proteins) and others with immunoregulatory activities (IRF1, IRF3 and MHC class I).^{2,12}

Recently we found that IFN- α 5 is the only IFN- α subtype which is expressed constitutively in the liver and that the level of IFN- α 5 mRNA is markedly decreased in the liver of patients with chronic hepatitis C virus (HCV) infection.¹³ At present, IFN- α therapy of chronic hepatitis C is based on the use of IFN- α 2. However this agent, even in the pegylated formulation and in association with ribavirin, fails to control viral replication in more than 50% of patients with

Steinheim, Germany) to verify equal loading of proteins. Membranes were incubated in TBS-T (50 mM Tris-HCl (pH 7.6) 200 mM NaCl, and 0,1% Tween-20) with 5 % dry milk. Proteins were detected by incubation with the specific antibody in TBS-T. After extensive washing in TBS-T, horseradish peroxidase-conjugated antibody was added for 1 h. Membranes were subjected to extensive washings in TBS-T and the specific protein bands were visualized using the enhanced chemiluminescence detection system (Perkin Elmer, Boston, MA), according to manufacturer's instructions. For reprobing, blots were stripped from membranes following the instructions of manufacturer. Membranes were autoradiographed and bands were quantified by densitometric analysis performed by Molecular Analyst/PC software (Bio-Rad laboratories, Inc).

Analysis of mRNA expression by quantitative real-time PCR . Total RNA was extracted from HepG2 cells using Ultraspec Reagent (Biotex, Houston, TX). One microgram of RNA was treated with DNase (Gibco-BRL, Paisley, U.K.) prior to reverse transcription with M-MLV Reverse Transcriptase (Gibco-BRL) in the presence of RNaseOUT (Gibco-BRL). MxA, p56 and 2'-5' OAS expression was measured by quantitative real-time PCR using a LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), and the LC-DNA Master SYBR Green mix. Aliquots of 3 µl from dilution 1/10 of the cDNA pool were used for each polymerase chain reaction (PCR), containing upstream and downstream primers specific for each gene (Table 1) in a 10 µl final volume. To determine the specificity, the PCR products were analyzed by melting curves. As an internal control for each sample, PCR amplification of a fragment of GADPH cDNA was performed. The amount of each transcript was expressed by the formula: $2^{cp(GADPH)-cp(gene)}$, being cp the point at which the fluorescence rises appreciably above the background fluorescence.

Statistical analysis

Normality was assessed with the Shapiro-Wilks test. Statistical analyses were performed using parametric (Student's *t*) and non-parametric (Kruskal-Wallis and Mann-Whitney *U*) tests. All *P*-values were two-tailed and considered being significant if the associated value was less than 0.05. Descriptive data for continuous variables are reported as means \pm SD or as medians and interquartile range. SPSS 9.0 for Windows was used for the statistical analysis.

RESULTS

STAT1-phosphorylation by IFN- α 5 and IFN- α 2: a comparative study in HepG2 cells

We compared the ability of IFN- α 5 and IFN- α 2 to activate STAT1, a critical step to initiate IFN-stimulated effects.¹⁶ HepG2 cells were starved in DMEM for 8 h prior to IFN exposure.

Following stimulation with IFN, cells were collected at different time points and cell lysates were analyzed by western blot with antibodies recognizing specifically STAT1 or the tyrosine phosphorylated form. Both IFN- α subtypes rapidly induced STAT1-tyr⁷⁰¹. However, IFN- α 5 induced stronger STAT1-tyr⁷⁰¹ signal than IFN- α 2 at 15, 30 and 60 minutes after stimulation (Fig. 1A). The densitometric values of the STAT1-tyr⁷⁰¹ band were about two times higher for IFN- α 5 than for IFN- α 2 at all these time points (Fig. 1B). Values at minutes 1 and 5 after stimulation with IFN were similar for the two IFN- α subtypes (data not shown). No differences with respect STAT1-Ser⁷²⁷ phosphorylation were observed after incubation of the cells with IFN- α 2 or IFN- α 5 (data not shown).

IFN- α 5 induces higher Tyk-2 phosphorylation than IFN- α 2 in HepG2 cells

Activation of Tyk-2 kinase, is essential to phosphorylate Tyr⁷⁰⁵ in STAT3. We compared the kinetics of Tyk-2 tyrosine phosphorylation induced by IFN- α 2 and IFN- α 5 in HepG2 using an antibody that recognizes Tyk-2 phosphorylation in 1054/1055 Tyr position. Induction of phospho-Tyk-2 from min 10 to min 60 following stimulation with IFN was more potent when cells were incubated with IFN- α 5 than when using IFN- α 2 (Fig.2A). Densitometric scanning showed that the p-Tyk-2 bands at min 10, 30 and 60 were, respectively, 4, 8 and 1.5 times more intense with IFN- α 5 than with IFN- α 2 (Fig.2B).

IFN- α 5 induces higher STAT3 tyrosine phosphorylation than IFN- α 2 in HepG2.

STAT3 is associated with the IFNAR1 subunit and it is phosphorylated by Jak-1/Tyk-2 kinases when cells are treated with IFN type I.^{3,9} We compared the ability of IFN- α 5 and - α 2 to activate this transcription factor in HepG2 cells by analyzing protein extracts from cells sampled before and at various time points after addition of IFN using specific antibodies for STAT3, STAT3-Tyr⁷⁰⁵ or actin. We performed experiments to determine the activation kinetics of STAT3-Tyr⁷⁰⁵ at early (1-30 min) and late (15-120 min) time points after stimulation with IFN (Figs 3A and 3B, respectively). IFN- α produced phosphorylation of STAT3 with a peak at 15 min either when using IFN- α 2 or IFN- α 5. However IFN- α 5 induced stronger activation signal of STAT3 from min 1 to min 60. Densitometric values of the STAT3-Tyr⁷⁰⁵ band were 1.5-2 times higher with IFN- α 5 than with IFN- α 2 at 1, 5, 15, 30 and 60 min after stimulation with IFN (Fig. 3A and 3B).

With respect STAT3 phosphorylation in Ser⁷²⁷ we observed a signal of low intensity in cells previously to the addition of IFN. Stimulation with either IFN- α 5 or IFN- α 2 did not induce any substantial change in the intensity of the STAT3-Ser⁷²⁷ signal with respect total STAT-3 protein contained in lysates (not shown).

Comparative analysis on the expression of IFN-inducible genes by IFN- α 5 and IFN- α 2.

We next studied whether the differences found between IFN- α 5 and IFN- α 2 on their ability to activate STAT1 and Tyk-2/STAT3 signaling pathways was paralleled, or not, by differences in their power to stimulate the expression of antiviral IFN-sensitive genes. Thus, we measured by real-time quantitative PCR the mRNA levels of 2'-5' OAS, MxA, and p56 in HepG2 cells at 14h after stimulation with IFN- α 2 or IFN- α 5. As shown in figure 4 both IFN- α subtypes were able to increase significantly the steady-state levels of mRNA of the three genes analyzed. However, as compared with IFN- α 2, IFN- α 5 induced significantly higher mRNA levels of the three genes, 2',5'-OAS ($p < 0.001$), p56 ($p < 0.001$) and MxA ($p < 0.007$).

DISCUSSION

IFN- α constitutes not a single molecule but a family of cytokines composed of at least 13 subtypes¹⁷ which show a close similarity at the structural level and exhibit an homology of 80-100 % in the amino acid sequence.¹⁸ All of them interact with the same receptor and induce similar biological effects including antiviral, antiproliferative and immunomodulatory activities.^{1,12} The reason for the presence of so many IFN- α subtypes with highly structural homology among them remains obscure. However recent data suggest that the sites that bind to the receptor may differ among subtypes and that some subtypes would bind to the receptor with higher efficiency than others.^{19,20} Moreover some reports have shown that the intensity of biological effects on specific cell types can be different for the diverse IFN- α subtypes.^{21,22} Thus, it seems that small variations in the primary sequence of the ligand may influence its interaction with the receptor resulting in differences in the transmission of the signal.²³

Examination of primary structure of IFN- α 2 and IFN- α 5 proteins reveals 85% of homology between them, with 24 differences in amino acid residues. Notably, it has been shown that Arg at position 22 of IFN- α 2 sequence is important with respect antiviral activity.²³ In IFN- α 5, position 22 consists of Gly (a non charged residue) instead of Arg (a positively charged residue), a change that could influence the electrostatic interaction with the receptor and intracellular signaling. Moreover it has been proposed that residues 24 to 29 of the IFN- α 2 sequence are involved in binding to the receptor.²³ The replacement of Leu (a hydrophobic residue) at position 26 of IFN- α 2 by a Pro (a hydrophilic residue) in IFN- α 5 may cause a change of the tridimensional structure of interferon that may modify the affinity for the receptor and the biological activity. Hence, we found reasonable to investigate whether IFN- α 2 and IFN- α 5 could exert different biological responses on hepatocytic cells.

The interest in comparing IFN- α 2 and IFN- α 5 with respect their effects on liver cells also stems from our previous finding that the only IFN- α subtype expressed constitutively in the liver is IFN- α 5.¹³ Interestingly the mRNA levels of IFN- α 5 are markedly decreased in chronic hepatitis C¹³ possibly reflecting a viral strategy to escape to the endogenous interferon system. Here we show that IFN- α 5 induces more potent activation of STAT-1, Tyk-2 and STAT3 than IFN- α 2. These differences in signaling are accompanied by a more intense induction of antiviral IFN-inducible genes. Thus the mRNA levels of p56, MxA and 2'-5' OAS in HepG2 cells after stimulation with IFN- α 5 are approximately two times higher than when cells were activated with IFN- α 2. Although it has been shown that IFN- α 8 is as potent as IFN- α 5 (and more than IFN- α 2) with respect protection against ECM virus in HuH7 cells,²² IFN- α 8, in contrast to IFN- α 5, is not naturally expressed in the normal nor in the diseased liver. Since IFN- α 5 is not available to treat liver patients it is not possible to compare *in vivo* its biological effects with those of other IFN- α subtypes in the whole ecosystem of the liver composed of a variety of cell types. However the fact that hepatic IFN- α expression is restricted to IFN- α 5 suggests, together with our *in vitro* findings, that this particular subtype may display more efficient antiviral activities in the hepatic tissue than other subtypes including IFN- α 2.

In conclusion, we show that IFN- α 5 induces stronger intracellular signaling and higher expression of antiviral interferon-inducible genes than IFN- α 2. Our data offer grounds for consideration of IFN- α 5, the IFN- α subtype expressed constitutively in the liver, as a possible alternative antiviral therapy for patients with liver disease.

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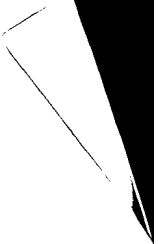
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Table 1. Primer sequences used in the study

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
2'-5' OAS	TTAAGAGGCAACTCCGATGG	AGCAGACTGCAAACCTCACCA
P56	ACCTGGGGCAACTTTGCCTGG	CAAAGCAGGCCTTGGC
MxA	ATCGGGGACCAGAG	ATGTAGCCCTTCTTCAG
GAPDH	GGTCGGAGTCAACGGATT	CCAGCATCGCCCCACTTGA

Legend to figures

Fig. 1 Differential induction of STAT1-tyrosine phosphorylation by IFN α -2 and IFN- α 5 .

A: Analysis of STAT1 tyr-phosphorylation. HepG2 were untreated (time 0) or stimulated with 5.000 units/ml IFN- α 2 or IFN- α 5 for indicated times. Immunoblot analysis of total cell lysates for each treatment was assessed with the anti-STAT1-Tyr⁷⁰¹- antibody. The membrane was stripped and presence of total STAT1 protein was determined by using an anti-STAT1 antibody. Corresponding samples were also examined for actin concentrations by using anti-actin antibody as protein loading control. **B:** Results are also expressed as the fold induction of the STAT1-tyr/STAT1 ratio for each sample compared with the ratio obtained under untreated conditions. Results are representative of three independent experiments.

Fig.2 Differential activation of Tyk-2 by IFN- α 2 and IFN- α 5. **A** HepG2 cells were starved for 8 h and then incubated in medium 2% FCS in the absence (time 0) or presence of 5000 units/ml IFN- α 2 or IFN- α 5 for the indicated times. An anti-Tyk-2 phospho-Tyr-specific antibody was used to determine the Tyk-2 phosphorylation state by using whole cell lysates. Membrane was sequentially stripped and reprobed with antibody against Tyk-2 protein. **B.** The blots were subjected for densitometry and phsopho-Tyk-2/Tyk-2 ratio changes relative to untreated cells are expressed as fold induction. Results are representative of three independent experiments.

Fig. 3. Differential induction of STAT3-Tyrosine phosphorylation by IFN- α 2 and IFN- α 5.

A Left panel: Kinetics of STAT3 tyr-phosphorylation after IFN- α 2 or IFN- α 5 stimulation at early time points. HepG2 cells were starved for 8 h and subsequently untreated (time 0) or treated with 5000 units/ml IFN- α 2 or IFN- α 5 for the times depicted in the Figure. Cell lysates were immunoblotted with phospho-Tyr-specific STAT3 antibody or anti-actin antibody as loading control. **Right panel:** results are also represented as fold induction of STAT1-Tyr/actin ratio relative to unstimulated samples. **B. Left panel:** Analysis of STAT-3 tyr-phosphorylation at later time points. HepG2 were untreated (time 0) or stimulated with IFN- α 2 or IFN- α 5 for indicated times. Immunoblot analysis of total cell lysates for each treatment was assessed with an anti-STAT3-Tyr⁷⁰⁵- antibody. The membrane was stripped and presence of total STAT3 protein was determined by using an anti-STAT3 antibody. Likewise, presence of actin protein was assessed by using an anti-actin antibody as loading control. **Right panel,** Results are also expressed as fold induction of the STAT3-tyr/STAT3 ratio compared with the ratio obtained under untreated conditions. Results are representative of three independent experiments.

Fig. 4 Differential antiviral gene induction between IFN- α 2 and IFN- α 5. 2'-5' OAS, p56 and MxA mRNA expression by real-time PCR in HepG2 unstimulated and stimulated for 14 h with IFN- α 2 or IFN- α 5. Results of 3 independent experiments in triplicate are represented

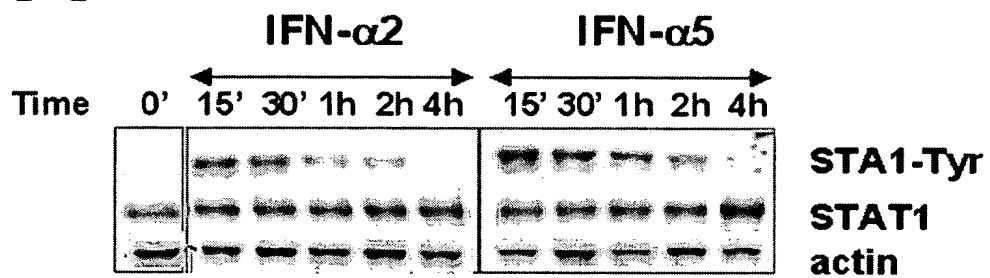
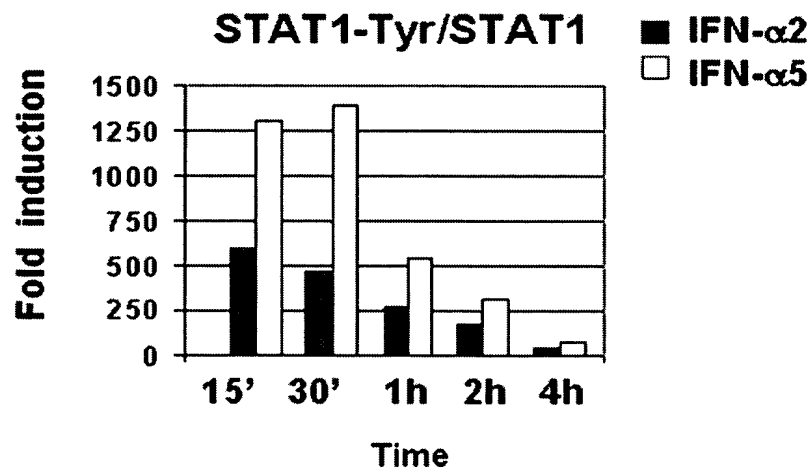
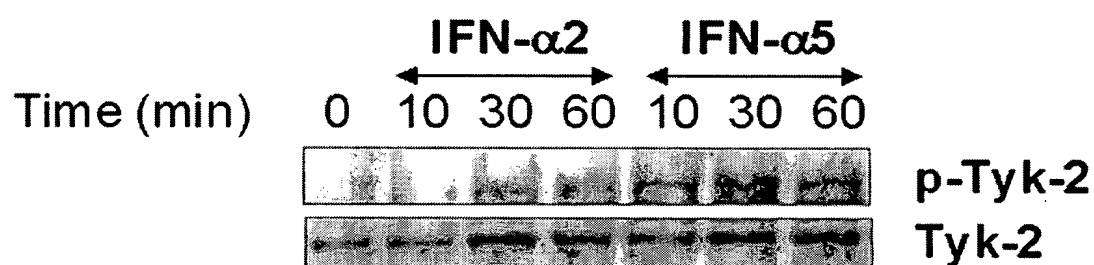
A**B**

Figure 1

A



B

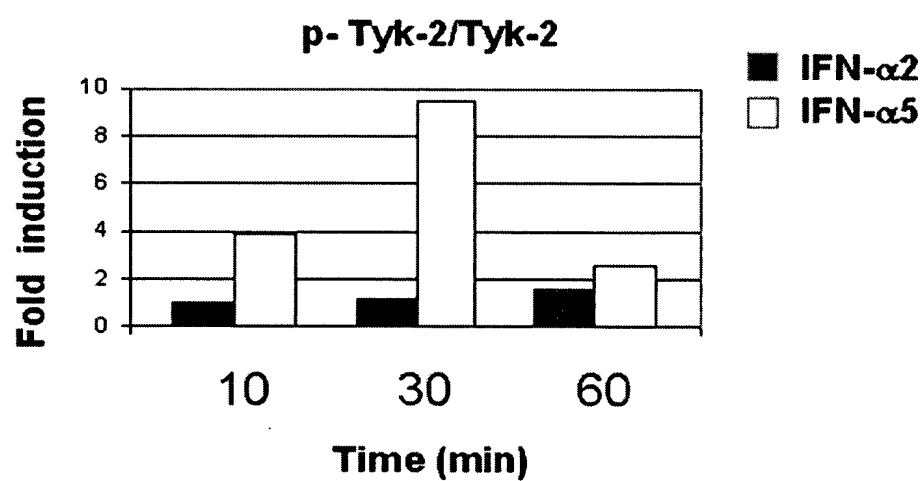
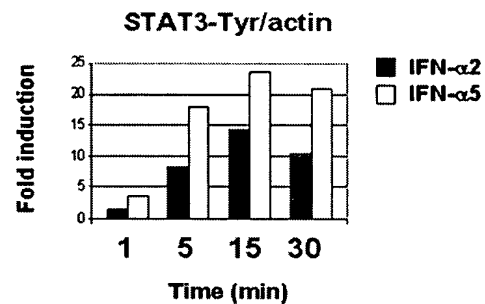
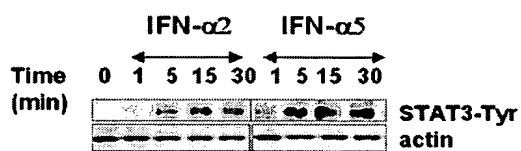


Figure 2

A



B

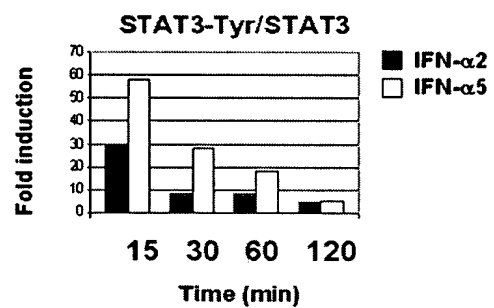
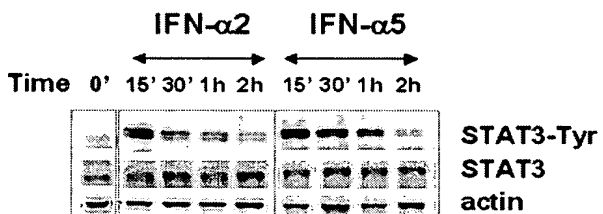


Figure 3

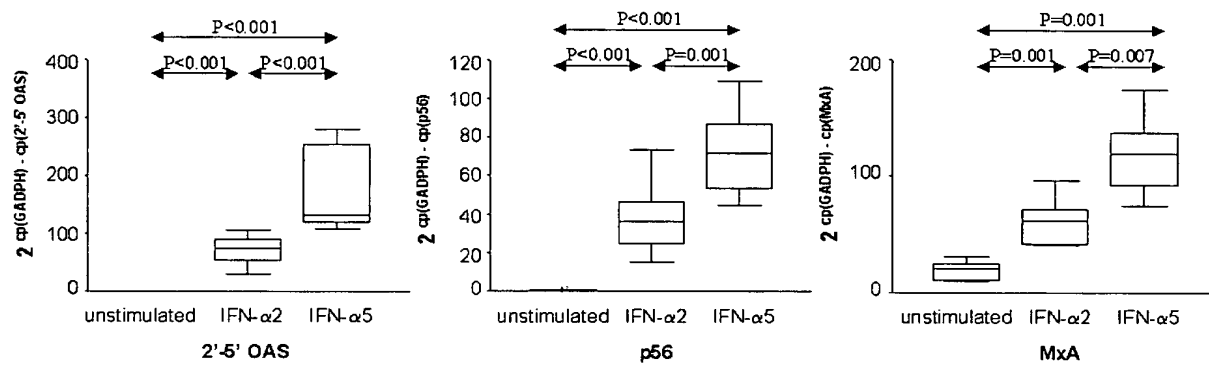


Figure 4